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Small-Angle Neutron Scattering Studies of Mixed Bile Salt-Lecithin Colloids

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Small-angle neutron scattering is used to analyze the morphology of particles formed in aqueous colloids of glycocholate with lecithin mixed at different lecithin to glycocholate molar ratios. Of interest are the transformations which occur when the system is diluted in aqueous solution. These are presumed to model the changes that occur in the formation of bile and when the colloid mixtures are used as novel drug delivery systems. It is observed that substantial changes in particle morphology occur with dilution. At high total lipid concentrations the particles are globular. As the system is diluted they elongate and at certain dilutions long rods are formed. At lower total lipid concentrations large bilayer sheets of undetermined shape and size are formed. Bilayer vesicles are observed at still lower total lipid concentrations, and become smaller with further dilution. The formation of rods with dilution is not in accord with the current theory of micelle structure and the mechanism of micelle growth in these systems. The relatively large solubility of glycocholate compared to that of lecithin may cause the former to repartition into the bulk solvent upon dilution, and thus induce the structural transformations noted above.

INTRODUCTION

Mixed aqueous colloids of bile salts and lecithin form particles with a variety of morphologies, depending on the amounts of each component and the type of bile salt present. The structural aspects of these colloids have been studied extensively, mostly using quasi-elastic light scattering (QELS)[1–5] and NMR.[7,8] The interest in these systems is due to their relevance in understanding the structure and action of bile, and their potential uses as novel drug delivery systems as well as model membranes. In the formation of bile, the components are initially mixed at high concentration, then diluted on passing into the upper intestine. The same basic process is involved in a drug delivery system in which lecithin, bile salt and a drug would be reconstituted at high concentration. Dilution occurs when the preparation is injected into the blood stream. Model membranes would be produced by a similar route. It is well known from previous studies[1–9] that dilution results in considerable changes in particle structure of the mixed colloids. Adequate structural models are needed if the important physical chemical principles in determining the particle structure are to be understood.

Previous work using QELS[1–5] has determined a preliminary structural phase map of this system. This work has shown that bile salt breaks up the propensity of the lecithin to form extended lamellar arrays. Discrete particles containing bile

salt and lecithin result. The particles are small when formed at high total lipid concentrations and when bile salt concentrations are sufficiently high. When the concentrated mixed colloids are diluted the structures increase in size up to a point. On further dilution the particles decrease in size. Although these studies have roughly characterized the transformations that occur in these systems, the structural parameters are based on analyses which in some cases may not apply to this system. Further, these methods probe the structures at very large, global scales that do not give structural information needed to understand the particle organization. More direct methods are required to give this information.

We have been using small-angle neutron scattering (SANS) to derive a structural phase map for these systems. The first of these studies[9] dealt with particle morphology in a dilution series of glycocholate with lecithin at a molar ratio (L/BS) of 0.56. In that study much greater structural detail of the particles was obtained than was available previously. In addition—and most important—it was shown that particle growth occurs by extension of initially globular micelles along a single axis to form long, rod-like particles which then transform into bilayer vesicles on further dilution. It was shown that the vesicles are initially heterogeneous in size and or shape (or that they are not entirely spherical). With further dilution, however, the populations are found to become structurally more homogeneous and spherical. Here, we review this work and describe additional SANS measurements on another lecithin-glycocholate mixture at L/BS = 0.9.

EXPERIMENTAL

Preparation of glycocholate-lecithin mixed colloids

Purified egg yolk lecithin (Sigma Biochemicals, Type VIIIe) and Sodium glycocholate (Sigma Biochemicals) were dissolved in ethanol. The solutions were mixed in the required molar ratios. The material was then dried *in vacuo* until the weight became constant. The preparation was dissolved in D_2O buffer containing 0.15 M NaCl, 20 mM tris (pH 7.5) to make a stock solution containing 50 mg/ml total lipid concentration. Samples at the final concentrations were prepared by a sequence of rapid dilutions with additional D_2O buffer. Samples were placed in vials which were then flushed with nitrogen and wrapped in foil to protect against light exposure. The samples were incubated for at least two days at 20°C.

Small-angle neutron scattering measurements

Samples in D_2O are placed in 2 mm pathlength cylindrical cells just prior to the measurement. The temperature was maintained at 20°C during the course of the measurement, and any unnecessary exposure to light was avoided.

SANS measurements were carried out on the Small-Angle Neutron Diffractometer (SAD) at the Intense Neutron Pulsed Source (IPNS) at Argonne National Laboratory. The instrument was configured with the detector in the head-on position for experiments with the lecithin/bile salt (L/BS) molar ratio of 0.56 and in the off-axis (4 cm) position for the L/BS = 0.9 samples. The later configuration

improves the Q-precision of the measurement.[10,11] Scattering data were acquired using time-of-flight, t, measurements taken at intervals of length Δt such that $\Delta t/t = 0.05$. Data are mapped into Q-space using procedures described elsewhere.[10–12] All data were placed on an absolute scale using the known cross section, $S(0)$, of an irradiated aluminum sample, Al-4[13] generously provided by W. Koehler of Oak Ridge National Laboratory.

Several analytical methods were used. A nonlinear least squares procedure was used to fit the scattering laws to those of ellipsoid and cylindrical models when applicable. Modified Guinier analyses were used to calculate the cross-sectional radius of gyration, R_c, and the excess total scattering cross section per unit length per unit weight of sample, $B\overline{m}_0$, for rod-like particles.[14] A similar analysis was used for sheet-like structures to derive the cross-sectional thickness radius of gyration, R_d, and the excess total scattering cross section per unit area per unit mass of sample, $B\overline{\mu}_0$.[15] Calculations of scattering expected from different structural models were done using standard analytical expressions,[16] and were smeared with the appropriate resolution function for the SAD.[11] All regression parameters are reported at 0.975 confidence intervals.

RESULTS

Lecithin-glycocholate mixed colloids: L/BS equals 0.56

Scattering from aqueous lecithin-glycocholate mixed colloids changes considerably with dilution (Figure 1). At the highest concentration studied (50 mg/ml) the form of the scattering curve suggests a globular particle. Interparticle effects are present, however, which make further analysis difficult (Figure 1A).

When the system is diluted to 10 mg/ml (Figure 1B), the scattering law suggests that the particles have become larger, and that interparticle effects have become less important. The scattering is fit by a nonlinear least squares procedure to an prolate ellipsoid (2a × 2a × 2b) with semi-axes, a, equal to 22 ± 2. Å and b, equal to 109 ± 15 Å (Figure 1B). Attempts to force a solution to an oblate ellipsoid give unphysical particle dimensions. The same result is obtained if cylindrical models are fitted to the data. This model, which assumes a homogeneous population, is probably not completely adequate, as the structures are likely to be polydispersed.

At lower concentrations (5 mg/ml; Figure 1C) the particle size has increased further. The curves suggest rod-like particles. This is confirmed by plotting the data in a modified Guinier analysis[14] (Figure 2). The resulting straight line implies $R_c = 19.2 \pm 0.1$ Å, and $B\overline{m}_0 = (3.58 \pm 0.04) \times 10^{-3}$ cm^{2}mg^{-1}Å^{-1}. If the particle is modelled as a uniform rod this value of R_c corresponds to a cross-sectional radius of 27.2 ± 0.2 Å. The correspondence between this value and that calculated for the a semi-axis of the prolate ellipsoid fit to the 10 mg/ml sample suggests that the particles present at that concentration have elongated on dilution to 5 mg/ml.

The process of enlargement with dilution continues to be observed in the next sample diluted to 2.5 mg/ml (Figure 1D). A plot of $\ln[Q^2 S(Q)]$ verse Q^2 is now linear (Figure 3), implying that the structure is now sheet-like. R_d is found to be

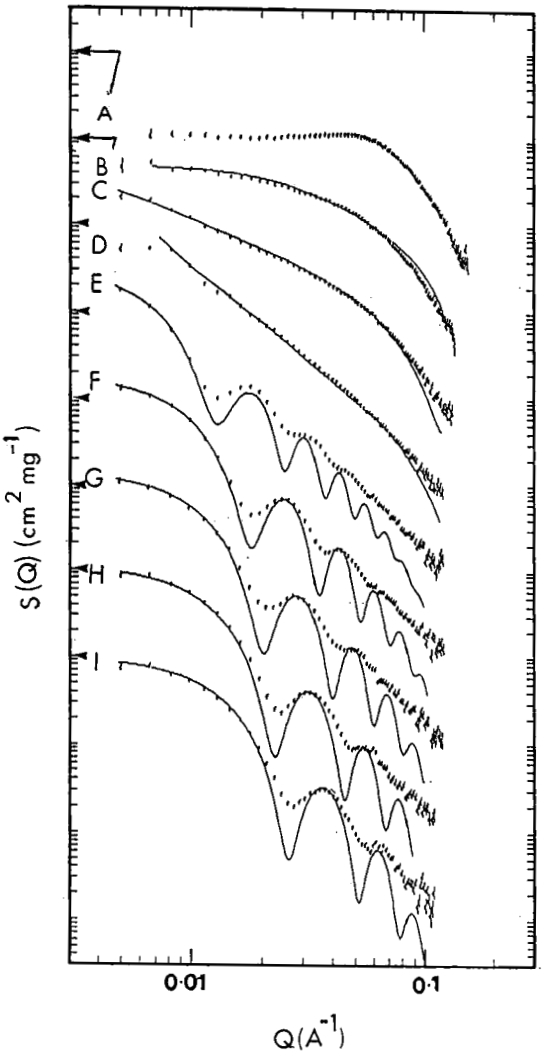

FIGURE 1 Scattering laws measured for mixed glycocholate-lecithin colloids with L/BS equal to 0.56, and comparisons with model calculations: Differential cross sections per mg of total lipid are plotted versus Q. The total lipid concentrations are: A, 50 mg/ml; B, 10 mg/ml; C, 5 mg/ml; D, 3.3 mg/ml; E, 2.5 mg/ml; F, 2.0 mg/ml; G, 1.67 mg/ml; H, 1.4 mg/ml; I, 1.0 mg/ml. Arrows indicate scales for the respective data: A–C, 1.0 cm²/mg; D–I, 10 cm²/mg. Data, ... Calculated scattering curves, — — — — — — , are for the models: B, a prolate ellipsoid with semi-axes 22.5 Å and 109 Å; C, an infinite rod with $B\overline{m}_0 = 3.36 \times 10^{-3}$ cm²mg^{-1}Å^{-1} and radius 27.2 Å; D, a large disk 32 Å thick with $B\overline{\mu}_0 = 5.91 \times 10^{-5}$ cm²mg^{-1}Å^{-2}; E, spherical vesicle 277 Å radius, 40 Å thick; F, spherical vesicle 202 Å radius, 44 Å thick; G, spherical vesicle 178 Å radius, 44 Å thick; H, spherical vesicle 162 Å radius, 44 Å thick; I, spherical vesicle 144 Å, 46 Å thick. Model calculations are smeared with the instrument resolution function.

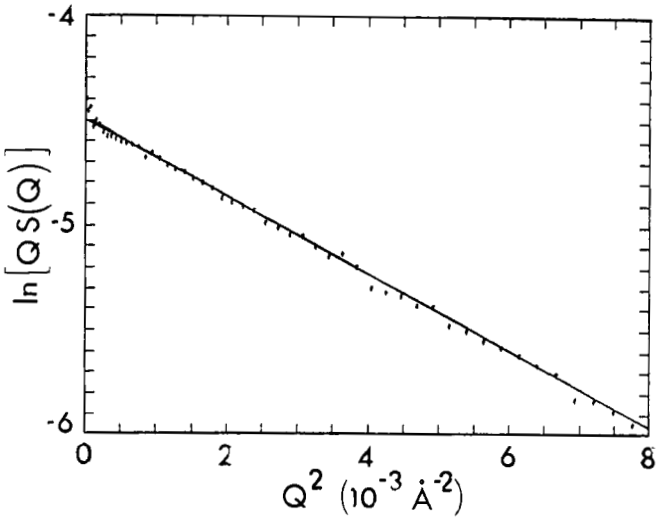

FIGURE 2 Modified Guinier analysis for the cross sectional structure of a rigid rod applied to the L/BS = 0.56, 5 mg/ml sample. .... , data; — — — — — — , least squares fit of a straight line: intercept = −4.49 ± 0.02, slope = −184 ± 3.

9.2 ± 0.2 Å, corresponding to a thickness of 32 ± 1 Å if the sheet is assumed uniform. $B\bar{\mu}_0$ is determined as (5.91 ± 0.02) × 10^{-5} $cm^2mg^{-1}Å^{-2}$. Figure 1 illustrates that the model of an infinite sheet does not fit the data at low Q. It must be concluded from this that the particle size is finite at the scales observed here. It is likely that the sheets are folded, probably into large, bilayer vesicles.

At the lowest concentrations studied the scatter observed is consistent with the

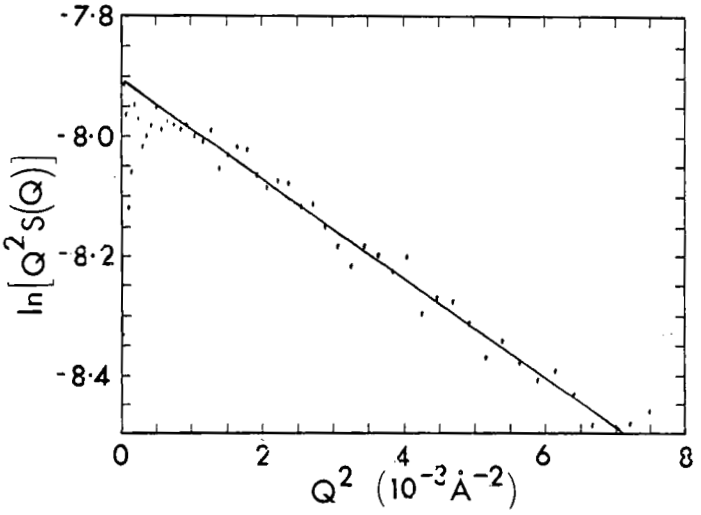

FIGURE 3 Modified Guinier analysis for the cross sectional structure of a sheet applied to the L/BS = 0.56, 3.3 mg/ml sample. .... , data; — — — — — — , least squared fit of a straight line: intercept = − 7.90 ± 0.03, slope = −84 ± 5.

presence of bilayer vesicles (Figure 1E–I). As the total concentration of lipids is decreased further the size of the vesicles decreases also. This is evidenced by the observation that the characteristic maxima move outward to larger values of Q with dilution, and that the differential cross section at very low Q becomes smaller, also. The bilayer nature of the vesicles is deduced from the likely thickness of the vesicle shell which is derived from the manner in which the intensity changes with Q. The change is relatively shallow and fits well to that expected for vesicles with walls about 40 A thick, a value expected for a single lipid bilayer in a D_2O solvent. Thicker shells, including solid spherical objects will have scattering laws that fall off much faster with Q.

Lecithin-glycocholate mixtures: L/BS equals 0.9

The SANS data for a dilution series at L/BS = 0.9 are shown in Figure 4. It is seen that the same morphological transformations observed in the L/BS = 0.56 case (Figure 1) are seen here also. There are significant differences, however. At the highest concentration (50 mg/ml) the particles are globular (Figure 1A). The interparticle interference effects, though still present, do not make as large a contribution to the low angle intensity as observed in Figure 1. Thus a shape analysis is possible. It is seen that the data can be equally well fit to an oblate or prolate ellipsoid (Figure 1A). For the oblate ellipsoid the semi-axes are $a = 39 \pm 1$ Å, $b = 19 \pm 4$ Å. The semi-axes for the prolate ellipsoid are $a = 26 \pm 3$ Å and $b = 47 \pm 14$ Å. We note that the value of a is the same as that observed for the cross-sectional radius of the rods observed in L/BS = 0.56, 5 mg/ml specimen (Figures 1C and 2).

In the next dilution to 20 mg/ml the analysis is less equivocal (Figures 4B and 5). The particle has clearly increased in size, as evidenced by the relative increase in $S(Q)$ in the low-Q part of the scattering curve. A least squares fit of the data gives a prolate ellipsoid with semi-axes $a = 21.5$ Å and $b = 159.6$ Å. Again, attempts to force a solution to an oblate ellipsoid resulted in particles with unphysical dimensions. The aspect ratio, $\gamma = b/a$, implied by this result, 7.4 ± 0.8, suggests that the modified Guinier analysis for rod-like particles is applicable,[14] and such an analysis gives $R_c = 19.0 \pm 0.2$ A and $B\overline{m}_0 = (3.96 \pm 0.05) \times 10^{-3}$ $cm^2mg^{-1}Å^{-1}$. The cross-sectional radius derived from this is 26.9 Å. It should be pointed out that the likely systematic errors of these parameters are estimated as -1.3 Å in R_c and -0.24×10^{-3} $cm^2mg^{-1}Å^{-1}$ in $B\overline{M}_0$ for rods with this value of γ.[14]

When dilution has been made to 10 mg/ml, long rods are once again present, as indicated by the linearity of the modified Guinier plot (Figure 5). The value of R_c calculated from the slope of the line is 19.1 ± 0.1 A, a value identical to that observed in Figure 2. $B\overline{m}_0$ is found to be $(3.78 \pm 0.02) \times 10^{-3}$ $cm^2mg^{-1}A^{-1}$. Comparison among the values observed for the 10 mg/ml and 5 mg/ml with L/BS = 0.56 and those observed in this series clearly indicates an underlying common organizational principle, in which the particles grow by elongation. At the higher L/BS ratio this growth occurs at lower dilution.

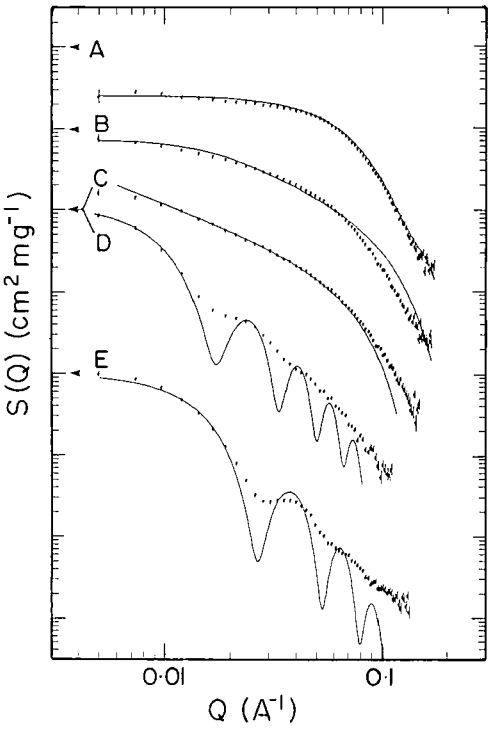

FIGURE 4 Scattering laws measured for mixed glycocholate-lecithin colloids with L/BS equal to 0.9, and comparisons with model calculations: Differential cross sections per mg of total lipid are plotted versus Q. The total lipid concentrations are: A, 50 mg/ml; B, 20 mg/ml; C, 10 mg/ml; D, 2.5 mg/ml; E, 1.67 mg/ml. Arrows indicate scales for the respective data: A–C, 1.0 cm/mg; D and E, 10 cm/mg. Data, ... Calculated scattering curves, — — — — — — — , are for the models: A, a prolate ellipsoid with semi-axes 26.3 Å and 47.1 Å, and an oblate ellipsoid 39.4 × 19.1 A; B, A prolate ellipsoid with semi-axes 21.5 Å and 159 Å; C, an infinite rod with $B\overline{m}_0 = 3.96 \times 10^{-3}$ cm^{2}mg^{-1}Å^{-1} and radius 26.9 Å; D, spherical vesicle 212 Å radius, 46 Å thick; E, spherical vesicle 145 Å radius, 45 Å thick. Model calculations are smeared with the instrument resolution function.

Spherical vesicles are formed when the serial dilution is made to 2.0 mg/ml (Figure 4D). At 1.4 mg/ml the sizes of the vesicles has decreased. Thus the two dilution series have these structural changes in common. There are some important characteristic differences between the two sets of data. First the scattering curves show features that are characteristic of greater polydispersity than the L/BS = 0.56 specimens. This is evidenced by the lack of definition between the different orders of scattering peaks. Second, for any given dilution the apparent size of the vesicle is smaller than the corresponding dilution in the L/BS = 0.56 samples. Because of the greater polydispersity it is more difficult to constrain the possible models consistent with the data. It appears however, that the shells of the vesicles have a larger apparent thickness in the L/BS = 0.9 than the preparation containing a lower percentage of lecithin. The apparent difference is likely to be a consequence of the simple, single step model used here to describe the structure of the wall.

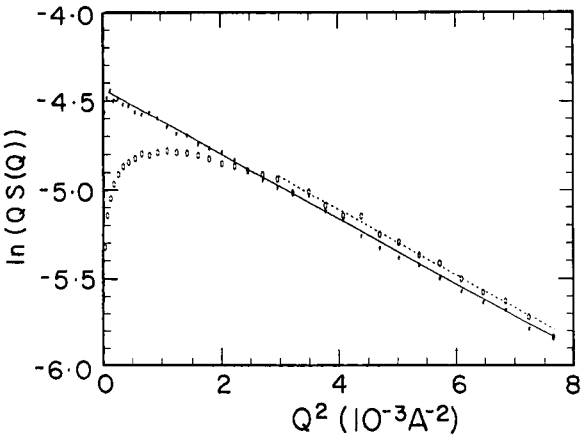

FIGURE 5 Modified guinier analysis for the cross sectional structure of a rigid rod applied to the L/BS = 0.9, 20 mg/ml and 10 mg/ml samples. .... , data for 10 mg/ml sample; — — — — — — — , least squares fit to a straight line: intercept = −4.43 ± 0.01, slope = −183 ± 2. ooooo, data for 20 mg/ml sample; - - - -, least squares fit to a straight line: intercept = −4.39 ± 0.04, slope = −181 ± 5.

The actual bilayer contains elements of different scattering length densities which are not in the same proportions in the two set of samples, and this may account for the observed differences. At the lowest concentrations used the data could not be fit satisfactorily. This may be the result of polydispersity, or may be a consequence of association between vesicles at this concentration and L/BS ratio.

DISCUSSION

Mixed colloids of lecithin and bile salts have been extensively characterized by QELS,[1-6] NMR[7,8] and other methods.[1,4] Regardless of this, the region in the structural phase map where the particles are seen to grow with dilution before undergoing a transition into vesicles is not at all well characterized. A model of the process has been devised[2] to account for the growth. In this model the micelles formed at high total lipid concentrations are envisioned as being disk-shaped particles. The micelle disks are thought to consist of a lecithin bilayer surrounded at its circumference by a ribbon of bile salt. The hydrophobic parts of the cholesterolic backbone of the bile salt interact with the fatty acid chains in the bilayer hydrophobic core. The hydrophilic parts of the molecule are then presented to the aqueous environment. Additional bile salt intercalates as dimers into the lipid bilayer. According to this picture, growth occurs with dilution as a result of extension of the structure in the plane of the disk. This is in response to repartitioning of the bile salt into the bulk solvent on dilution. Growth is then the result of less bile salt being available in the particle to form a ribbon around the lecithin disk. The ratio of area to circumference is inversely proportional to the disk radius. Hence, the larger the disk the smaller the proportion of bile salt needed to solubilize it.

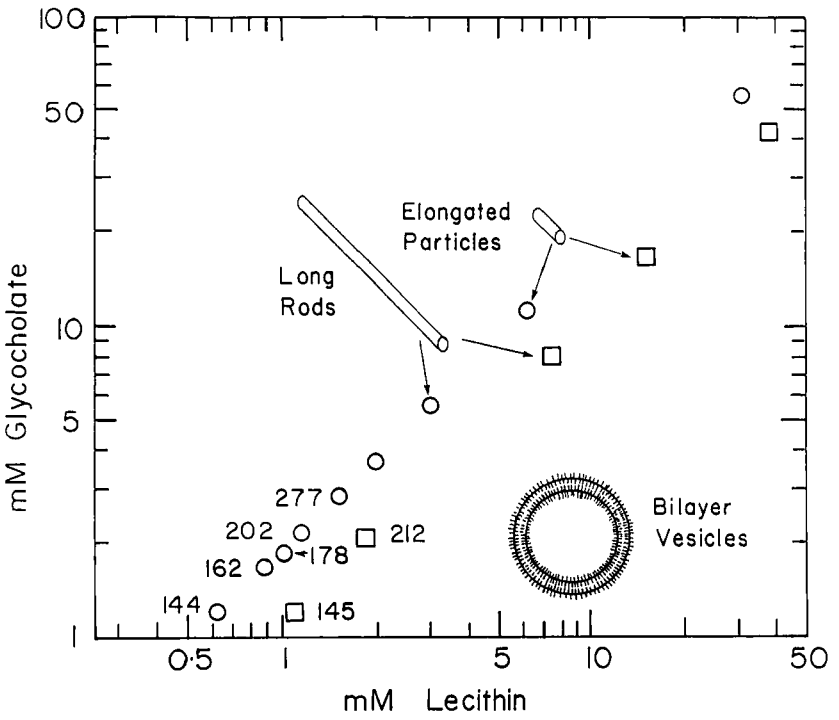

FIGURE 6 Summary of findings from SANS studies of dilutions of mixed glycocholate-lecithin colloids: The structural finding of SANS studies is presented as a function of the concentrations of lecithin and glycocholate in mM. oooo, L/BS = 0.56; L/BS = 0.9. In the most concentrated samples the particles are likely to be globular. The analysis, however, is complicated by the presence of interparticle interference effects in the scattering laws. At low concentrations the particles in both dilution series become elongated with a characteristic cross-sectional radius of 27 Å. The elongated particles become long rods with cross-sectional radius 27 Å with further dilution. At the lowest concentrations studied the particle are single bilayer mixed vesicles of lecithin and glycocholate; these are identified by points labelled with the apparent radii given in Å. The circle without any structural identification represents a sample either which has a sheet-like organization, but which may be a large vesicle.

The SANS results presented here—and summarized in Figure 6—for preparations containing two different L/BS molar ratios do not meet the expectations of this theory. The data show the presence of a population of elongated particles (Figures 1, 2, 4 and 5), even at concentrations as high as 20 mg/ml (Figures 4B and 5). Further, the evidence shows that these transform into long rods with further dilution (Figures 1, 2, 4 and 5). There must be a common organizational theme in the formation of these structures, as in every case considered the radius of the rods and the intermediate elongated particles appear to be about 27 Å. The maximum length of the hydrocarbon portions of the lecithin is about 30 Å. Thus, in principle the rods could accommodate a radial arrangement of the lecithin alkyl chains. This, however, is unlikely in that it is difficult to constrain lecithin in such an arrangement. It is more likely that the rods are produced by stacking of basic disk-like units structures similar to those described in the earlier work cited previously.[1-5] In this model stacking would result in stabilization of the bile salt-lecithin interactions.

The particle populations would be described as consisting of rods of different lengths.

The formation of micelles into rod-like aggregates is known in some simple micellar systems.[5,17] In the case of simple bile salt micelles[5] the growth has also been modelled as a linear aggregation of simple disk-like micelles. However, in each of these cases growth is associated with increases in total lipid concentration. Thus, the mechanism of rod formation in the mixed colloid is likely to be different from that in the simple micellar systems.

One question not addressed by the present work is the transformation to vesicles. It is clear from the dilution series at L/BS = 0.56 that rods transform into large vesicles or sheets. Perhaps the rods aggregate side by side and then fuse as rafts to form the sheets. The data do not access the full extent of such structures, however.

Whatever be the mechanism of vesicle formation, it is clear that the sizes of the vesicle, once formed, decrease with further dilution. This is undoubtedly the result of further leaching of glycocholate incorporated into the vesicle surface into the bulk solution. The resulting loss in surface area causes the vesicles to shrink. It is interesting to note that the vesicle radii may correlate with the concentration of glycocholate (Figure 6).

Acknowledgments

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